

# Identification of a 37 Kilodalton Protein at the Epidermal Basement Membrane by an Antiserum to Human Amnion

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A protein which is recognized by an antibody to human amniotic epithelial basement membrane was identified at the basal lamina of human epidermis by immunohistology. This protein was localized at the lamina lucida of human epidermal basement membrane by immunoelectron microscopy. Studies of normal human keratinocyte cultures and epidermal wound healing suggested that the protein

was probably produced by keratinocytes. By immunoblotting, a basic apparent isoelectric pH ( $pH_{iapp} = 7.3$ ) protein band of 37 kD was seen. These data indicate that this 37 kD protein, clearly different from other known basement membrane components, is present in simple and stratified epithelia of ectodermal origin, and is associated with hemidesmosomes. *J Invest Dermatol* 87:190-196, 1986

**B**asement membranes (BM) are morphologically defined extracellular structures that separate parenchymal, epithelial, and endothelial cells from the connective tissue proper. Although many questions remain to be answered concerning the biochemistry of BM, their major constituents have been characterized. This is the case for the epidermal BM that plays an important role in the dermal-epidermal interactions. Current information indicates that the dermal-epidermal junction is composed of collagens (type IV and perhaps type V), noncollagenous glycoproteins and proteoglycans, such as laminin, heparan sulfate-containing glycoprotein, and fibronectin [1]. Other components of BM include antigens identified by autoantibodies from bullous disorders (herpes gestationis and scarring pemphigoid) as well as bullous pemphigoid (BP) antigen [2].

Similar to epidermal keratinocytes, human amniotic epithelial cells are ectodermal, albeit extraembryonic in origin, and it has been shown that amniotic cells manifest unique and characteristic ectodermal antigens [3]. Antigenic similarities between amniotic epithelium and certain ectodermally derived tissues including Hassal's corpuscles of thymus [4], mammary glandular and corneal epithelia [5] have been demonstrated. On the other hand, it

has been shown that the BM zone of the amniotic epithelium reacted with BP antibodies by indirect immunofluorescence (IF) technique [6].

In this report, we demonstrate that a 37-kD protein which immunoreacted with an antibody to human amniotic epithelial BM was localized in the basal lamina of human epidermis. This protein, which is apparently distinct from other known BM components, is present in simple and stratified epithelia of ectodermal origin.

## MATERIALS AND METHODS

**Tissues** Full-term human amniotchorions and placentae were collected from normal pregnancies during vaginal deliveries at St-Roch Hospital, Nice, France. Human skins and thymuses were obtained during open heart surgery. Corneas were donated by the Eye Bank. Other tissues were obtained from autopsies which included vagina, cervix, thyroid, myocardium, adrenal gland, liver, brain, lung, kidney, testis, and spleen. Suction blisters from 2 women volunteers raised according to the procedure of Kiistala and Mustakallio [7] were biopsied together with the surrounding skin. For the study of cutaneous wound healing, an initial biopsy was performed using a punch of 3 mm diameter, then left covered with a gauze for periods of time varying from 1-6 days. A second biopsy including the wound and the surrounding normal skin was then performed using a punch of 5 mm diameter. Animal tissues were obtained from mouse, rat, guinea pig, rabbit, dog, pig, and monkey. These included: cornea, lip and oral mucosa, esophagus, vagina, cervix, skin, brain, muscle (cardiac, striated and smooth), blood vessels, lung, thyroid, adrenals, liver, pancreas, spleen, kidney, and testis. One-cubic-centimeter tissues were wrapped with aluminum foil and snap-frozen in liquid nitrogen. Amniotchorions and corneas were covered on 2 sides with thin slices of fresh mouse liver before freezing. All snap-frozen materials were stored at  $-20^{\circ}\text{C}$  until use.

**Cell Cultures** Human epidermal keratinocytes were obtained from adults during abdominal surgery and were cultured in Dulbecco's modified Eagle's medium (DMEM) (KC Biological Inc., Lanexa, Kansas) containing 10% fetal calf serum,  $1.1 \times 10^{-6}$  M hydrocortisone (Fluka, Buchs, Switzerland), and  $1.6 \times 10^{-9}$  M

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### Abbreviations:

- AEBM: amniotic epithelial basement membrane
- BM: basement membrane
- BP: bullous pemphigoid
- 2D: two-dimensional
- FITC: fluorescein isothiocyanate
- HRP: horseradish peroxidase
- IF: immunofluorescence
- PBS: phosphate-buffered saline
- PMSF: phenylmethylsulfonyl fluoride
- SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- TRITC: tetramethyl rhodamine isothiocyanate
- UEA 1: *Ulex europaeus* agglutinin 1 lectin

epidermal growth factor (Clinisciences, Paris, France) according to either the method of Liu and Karasek [8] or that of Rheinwald and Green [9]. Cell homogenates were made 3–5 days after they reached confluency. Pure epidermis homogenates were performed from 5–8 epidermal blister roofs (19 mm<sup>2</sup> each).

### Antibodies

**Anti-*Amnion* Sera:** The solubilization of amnion membranes and the production of the rabbit antiserum were detailed by Hsi et al [10]. The antiserum, called 6/2, was collected by immunizing and boosting twice with the first peak of Biol-Gel P-200 (BioRad, Richmond, California) chromatographed solubilized amnion. The prebled samples from the rabbit and the sera from 2 rabbits that were given 0.15 M phosphate-buffered saline (PBS) and 0.15 M NaCl were used as control sera. All sera were heat-inactivated, absorbed with pooled red blood cells and placenta powder according to Hsi et al [5]. IgG fractions of antiserum 6/2 were isolated by anion exchange chromatography with the use of DEAE-resin (Whatman Ltd., Maidstone, Kent, England). Protein concentrations obtained were close to 7 mg/ml.

**Other Antisera:** Autoantibodies were obtained from 6 patients with BP and 1 patient with herpes gestationis. By direct IF, all BP sera contained IgG antibodies which bound to the epidermal BM zone of normal human skin at titers higher than 1:40. Antilaminin antiserum was a kind gift of Dr. M. Prunieras, C.I.R.D. Valbonne. Antisera to fibronectin and type IV collagen were obtained from Institut Pasteur (Lyon, France). Fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin 1 lectin (UEA 1, M2) was purchased from Vector Laboratory (Burlingame, California) and used to identify endothelial cells [11]. A monoclonal antibody to neurofilament was the kind gift of Dr. Denise Paulin (Institut Pasteur, Paris).

**Immunohistology** Cryostat sections (4.5  $\mu$ m) were mounted on microscopic slides and air-dried at room temperature. The following procedures were performed at 4°C. Each section was incubated with 50  $\mu$ l of an optimal dilution of antiserum in 0.15 M PBS, pH 7.2, for 20 min, followed by 2 15-min washes in PBS. For IF, the sections were incubated with 50  $\mu$ l of 1:40 dilution of FITC-conjugated sheep antirabbit immunoglobulin for 20 min, and washed 3 times in PBS. Some sections were counterstained with propidium iodide according to Ockleford et al [12]. They were then mounted in PBS-buffered glycerol. Double-labeling experiments were performed with the following conjugates: tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit antimouse immunoglobulins (NORDIC, Tilburg, The Netherlands) and TRITC-conjugated swine antirabbit immunoglobulins (Dakopatts, Copenhagen, Denmark). All preparations were examined by epi-illumination with a Zeiss Universal microscope. Photographs were taken with a Zeiss MC63 camera system and Kodak Ektachrome 400 films.

For immunoperoxidase, sections were incubated with 30  $\mu$ l of dilution of horseradish peroxidase (HRP)-conjugated goat antirabbit immunoglobulin (Dakopatts) for 20 min, washed 3 times in PBS, and developed by adding 50  $\mu$ l of 3,3'-diaminobenzidine (0.5 mg/ml) with 0.1% H<sub>2</sub>O<sub>2</sub> to each section for 5–10 min, rinsed with water, and mounted in PBS-buffered glycerol. Photographs were taken with Ektachrome 64 films.

**Immunoelectron Microscopy** Fourteen micron-thick sections of snap-frozen normal human skin biopsies were fixed with 3% paraformaldehyde in PBS for 15 min, washed 15 min in PBS, and incubated with 300  $\mu$ l of 1:30 dilution of 6/2 serum at 37°C for 45 min according to MacDonald et al [13]. The sections were washed with PBS for 15 min, incubated with 50  $\mu$ l of 1:50 dilution of HRP-conjugated swine antirabbit immunoglobulin (Dakopatts) for 45 min at 37°C, and washed for 15 min. Then the sections were postfixed with 2% glutaraldehyde in cacodylate buffer for 20 min. The peroxidase activity was then revealed by 50  $\mu$ l of 3,3'-diaminobenzidine (0.5 ml) and 0.1% H<sub>2</sub>O<sub>2</sub> for 15

min. After washing in Tris-HCl buffer, pH 7.6, for 15 min, the sections were postfixed again with 1% osmium tetroxide for 10 min, washed, dehydrated in ethanol, and embedded in epoxy medium. Some ultrathin sections were counterstained with lead citrate and uranyl acetate. The samples were examined with a Hitachi HU 12A electron microscope. A negative control with a nonimmune rabbit serum was also performed.

**Monodimensional Gel Electrophoresis and Immunoblotting** Cultured human keratinocytes or isolated epidermis were washed several times with ice-cold PBS. They were then broken at 4°C using a Kontes glass-glass homogenizer in PBS containing 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and the resulting homogenate was sonicated for 15 s at 4°C. Protein contents of homogenates estimated by the method of Lowry et al [14] were in the range of 4–7.5 mg/ml. Aliquots of these homogenates (50–150  $\mu$ g of proteins) as well as standard molecular weight proteins (Pharmacia, Uppsala, Sweden) were subjected to a sodium dodecyl sulfate (SDS) denaturing polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli [15]. The acrylamide concentration of the resolving gel was 5% or 10% (see Figs 1–9) and electrophoresis was performed at 60–75 mA/gel (1.5 mm  $\times$  18 cm  $\times$  20 cm) for 4–5 h. At the end of running, one part of the gel was stained with Coomassie blue, whereas the other part was pre-equilibrated for 20 min by shaking with the following blotting buffer: 20 mM Tris-base, 150 mM glycine, and 20% (v/v) methanol. Then the proteins were transferred to nitrocellulose paper (0.45  $\mu$ m, BA 85 type, Schleicher & Schuell, Dassel, West Germany), with a transblot cell (BioRad), at 55 V and 4°C overnight using the blotting buffer as described above [16]. In order to verify the efficiency of transblotting and to stain the transferred standard proteins, one part of the nitrocellulose sheet was cut into strips and stained with 0.2% Ponceau red, whereas the other part was saturated for 90 min at room temperature by shaking with a solution of PBS containing 3% bovine serum albumin (fatty acid-free and globulin-free No. A 7030, Sigma, St. Louis, Missouri) and 0.2% Triton X-100 (assay buffer) [17]. The nitrocellulose paper was then cut into strips and reacted with either the non-immune serum or with the 6/2 serum (diluted with assay buffer) for 3 h at room temperature under shaking. After 4 1-min washes with assay buffer, the antibodies bound to proteins were further reacted with 1:500 dilution of HRP-conjugated antirabbit IgG for 45 min at room temperature. After several 2–3 min washes with assay buffer, the nitrocellulose strips were rinsed 3 times with PBS and the complexes were revealed with diaminobenzidine (0.5 mg/ml) in 0.1 M Tris-HCl pH 7.6 buffer containing 0.1% H<sub>2</sub>O<sub>2</sub>.

Blotting experiments carried out with collagen IV (the kind gift of Dr. Herbage, Centre Technique du Cuir, Lyon, France), human fibronectin (Institut J. Boy, Reims, France), and laminin (the kind gift of Dr. Bernard, C.I.R.D., Sophia Antipolis, France) were performed as above, except that respective antibodies were reacted 1.5–2 h. Anticollagen IV serum was a gift of Dr. Hartmann, Centre de Radioanalyse, Lyon, France.

**Two-Dimensional Gel Electrophoresis and 2-Dimensional Immunoblotting** Two-dimensional (2D) gel electrophoresis was performed according to O'Farrell [18]. Keratinocytes grown on 78-cm<sup>2</sup> culture dish were washed several times with ice-cold PBS and homogenized in the following buffer: Tris 56 mM pH 6.8, 6.6% (w/v) Triton X-100, 0.19 M  $\beta$ -mercaptoethanol, and 5 M urea. Thereafter, samples containing 200–400  $\mu$ g proteins were laid on 2.5  $\times$  135 mm gel rods (gel electrophoresis cell BioRad, Richmond, California). A prerun was performed to establish the pH gradient obtained with pH 3.5–10 ampholines (Pharmacia) when 74 mM ethylene diamine was used as catholyte and 36.6 mM sulfuric acid was used as anolyte. Protein focusing was accomplished at 2400 volt-hours. Approximate determination of pH gradient along the focused gel rods was made by a contact pH electrode, standard proteins (Isoelectric Focusing Calibration

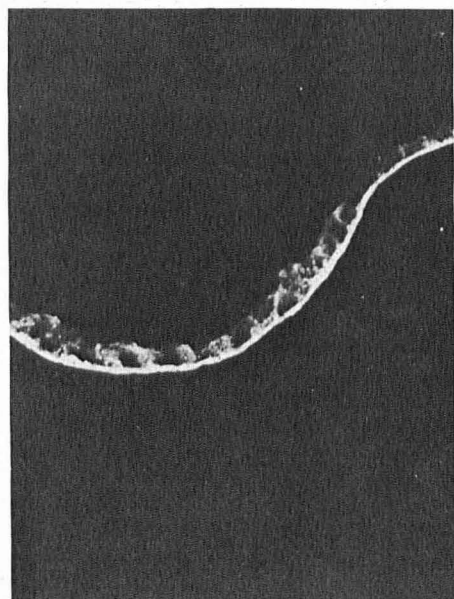
Kit, Pharmacia) as well as by measuring pH of water aliquots where portions of gel rods have been previously extracted. After focusing, the gel rods were rinsed 15 min with water and equilibrated 45 min into 62.5 mM Tris-Cl pH 6.8, containing 58% (w/v) glycerol, 2.5% (w/v) SDS, 0.68 mM  $\beta$ -mercaptoethanol, and 0.1% bromophenol blue. The 2D gel electrophoresis as well as subsequent immunoblotting were performed as described for monodimensional gels.

## RESULTS

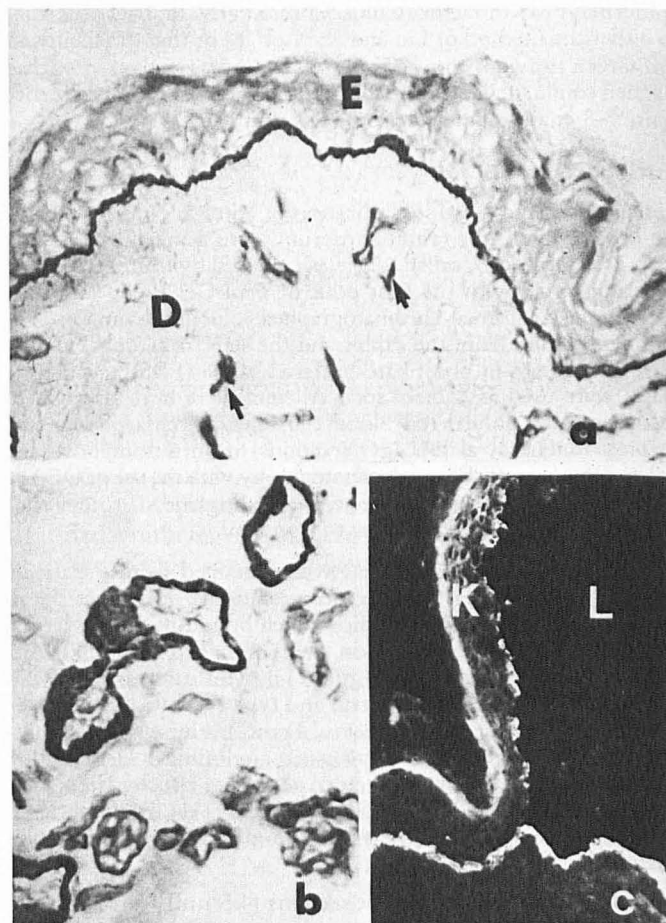
### Immunohistologic Studies

**Normal Skin:** The antiserum reacted brilliantly with the amniotic BM (Fig 1), the epidermal BM, and with the BM surrounding epidermal adnexae (Fig 2a,b) which included hair follicles, sebaceous glands, apocrine, and eccrine glands. The pattern was regular and continuous. In some sites, it appeared to be thicker. The prebleeds and control sera gave negative reactivities. The reactivity was observed using the antiserum after preabsorption on human amnion or human skin. Double-labeling experiments using TRITC-labeled 6/2 serum and the FITC-conjugated lectin UEA 1 showed that the BM surrounding dermal blood vessels were not reactive. A similar observation applied to dermal nerve endings as shown by double-labeling experiments using 6/2 serum and a monoclonal antibody against neurofilaments. The preincubation of skin sections with 5 BP sera and 2 herpes gestationis sera did not block the reactivity of 6/2 serum. Conversely, the pretreatment of skin sections with 6/2 serum did not block the reactivity of any BP sera or the deposition of C3 at the dermal-epidermal junction. The 6/2 serum showed a broad species specificity as positive reactivities were obtained on skin from mice, rats, guinea pigs, pigs, dogs, cats, and monkeys. Furthermore, the reactivity of 6/2 serum at the dermal-epidermal junction is independent of the type of epidermal differentiation. This was suggested by the studies that both ortho- and parakeratotic epidermis in rat tail and 3 different types (mucosal, orthokeratosis, parakeratosis) of epithelial differentiation in rabbit lip were recognized by 6/2 serum (data not shown).

**Keratinocyte Cultures:** All cells from freshly prepared epidermal cell suspensions were nonreactive with 6/2 serum, but were reactive with BP sera by double-labeling IF. However, the antigen detected by 6/2 serum was detectable as early as 2–3 days after



**Figure 1.** The reactivity of 6/2 serum on human amnion by indirect IF. Reactivities can be identified on the amniotic basement membrane as well as within the amniotic epithelial cells ( $\times 100$ ).



**Figure 2.** The reactivity of 6/2 serum on human skin. a, Linear staining of the dermal-epidermal junction by indirect immunoperoxidase. The basement membranes (arrows) surrounding the epidermal adnexae were also stained. E = epidermis; D = dermis ( $\times 100$ ). b, Higher magnification of the labeling of the basement membrane surrounding the eccrine sweat glands ( $\times 250$ ). c, The floor of the suction blister as well as the basal aspect of epidermal keratinocytes were reactive by indirect IF. L = lumen of the blister ( $\times 100$ ).

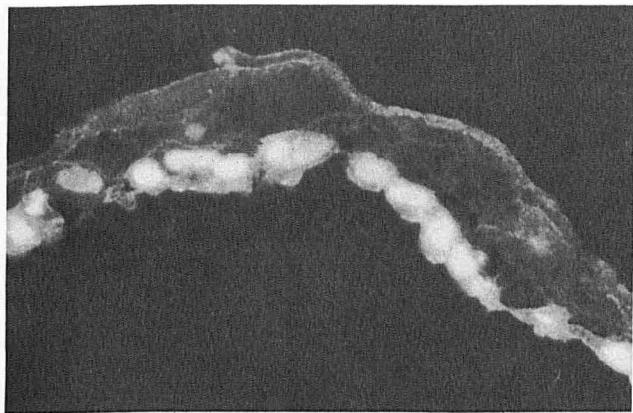
confluence in keratinocyte culture. It was not expressed in a linear pattern at the base of the cell layers, but was identified in the intercellular space, and many cells showed mainly bright cytoplasmic reactivities (Fig 3).

**Suction Blisters and Wound Healing:** On suction blisters (Fig 2c), 6/2 serum showed focal reactivities with the roof of the blisters and a linear pattern on the blister floor. The splitting occurred at the lamina lucida level, which was confirmed by the use of antisera against laminin, collagen IV, and BP sera (data not shown). For epidermal wound healing, the antigens detected by 6/2 serum were expressed beneath the lip of the migrating epithelium, whereas laminin and collagen IV were not present in the newly synthesized basement membrane. The BP antigen was detected in a similar location as the antigen recognized by 6/2 serum.

**Other Tissues:** The specificities of 6/2 serum were studied mainly on tissues from mice and rabbits as shown in Tables I and II. They were reactive with the BM of ectodermal-derived tissues which included oral mucosa, lip, cornea, vagina, cervix, breast, and esophagus.

**Immunoelectron Microscopic Analysis** Ultrastructurally, specific reactivity indicated the binding of 6/2 serum to the lamina lucida of the dermal-epidermal junction (Fig 4). No electron-dense deposits were found in the subbasal lamina area. In some





**Figure 3.** The reactivity of 6/2 serum on cultured normal human keratinocytes by indirect IF. Strong staining can be identified in the cytoplasm of the basal layer of the cultured keratinocytes. The section was counterstained with propidium iodide to highlight the nuclei ( $\times 250$ ).

parts where the staining was less intense, reaction products were not linearly distributed and deposits were separated by unstained zones. Immunoreactant was apparently related to hemidesmosomes (Fig 4b). A similar staining was observed surrounding hair follicles, sebaceous and sweat glands. In contrast, there was no specific binding of 6/2 serum to the BM surrounding dermal blood vessels and dermal nerve endings (data not shown). No specific reactivity was observed on the sample treated with the nonimmune rabbit serum (Fig 4a).

#### Identification of the Antigen Recognized by 6/2 Antiserum

**Monodimensional Gel Electrophoresis Analysis:** To identify the antigen recognized by 6/2 antiserum, immunoblotting technique was used. The electrophoretic pattern on monodimensional SDS-PAGE obtained from cultured human keratinocytes as well as from pure human epidermis are shown in Fig 5. Among these various bands related to cultured cells, antiserum 6/2 recognized a doublet at 37–38 kD, which was more strongly labeled by immunoreaction at 37 kD (mean of several determinations) (Fig 6). This doublet was barely seen in the case of suction blister roof of pure epidermis. This is in agreement with the morphologic data (Fig 2c), where the antigen was not found in great amount in the blister roof. Depending on keratinocyte culture series used in the course of experiments the doublet immunostaining appeared either distinct as shown in Fig 6, or less resolved, or even

**Table I.** Comparison of Reactivities of 6/2 Serum and Antilaminin, Anticollagen IV, and Antifibronectin Sera on Amniochorion, Skin, and Kidney Using IF Technique

Tissues	Antigen			
	"6/2 Antigen"	Laminin	Collagen IV	Fibronectin
Amniochorion				
Amniotic epithelium	+	+	+	+
Blood vessels	—	+	+	+
Skin				
Dermal-epidermal junction	+	+	+	+
Epidermal adnexae	+	+	+	+
Blood vessels	—	+	+	+
Nerves	—	+	+	+
Kidney				
Glomerules	—	+	+	+

**Table II.** Tissue Antigen Distribution and Species Specificity Related to 6/2 Serum by IF Technique

Tissues	Species		
	Mouse	Rabbit	Human
Oral mucosa	+	ND <sup>a</sup>	+
Lip	ND	+	+
Cornea	ND	+	ND
Vagina	+	+	ND
Cervix	+	+	ND
Breast	+	+	+
Esophagus	ND	+	+
Kidney	—	—	—
Liver	—	—	—
Lung	—	—	—
Spleen	—	—	—
Muscle	—	—	—
Myocardium	—	—	ND
Aorta	ND	—	ND

<sup>a</sup>ND, Not done, the oral mucosa of rat and esophagus of monkey were positive with 6/2 serum sera.

as a single band. Whatever the situation encountered, the peroxidase labeling in immunoblot was always stronger or unique at the 37 kD band. Absorption of 6/2 antiserum with pieces of skin resulted in a lack of immunostaining of the 37–38 kD doublet (data not shown).

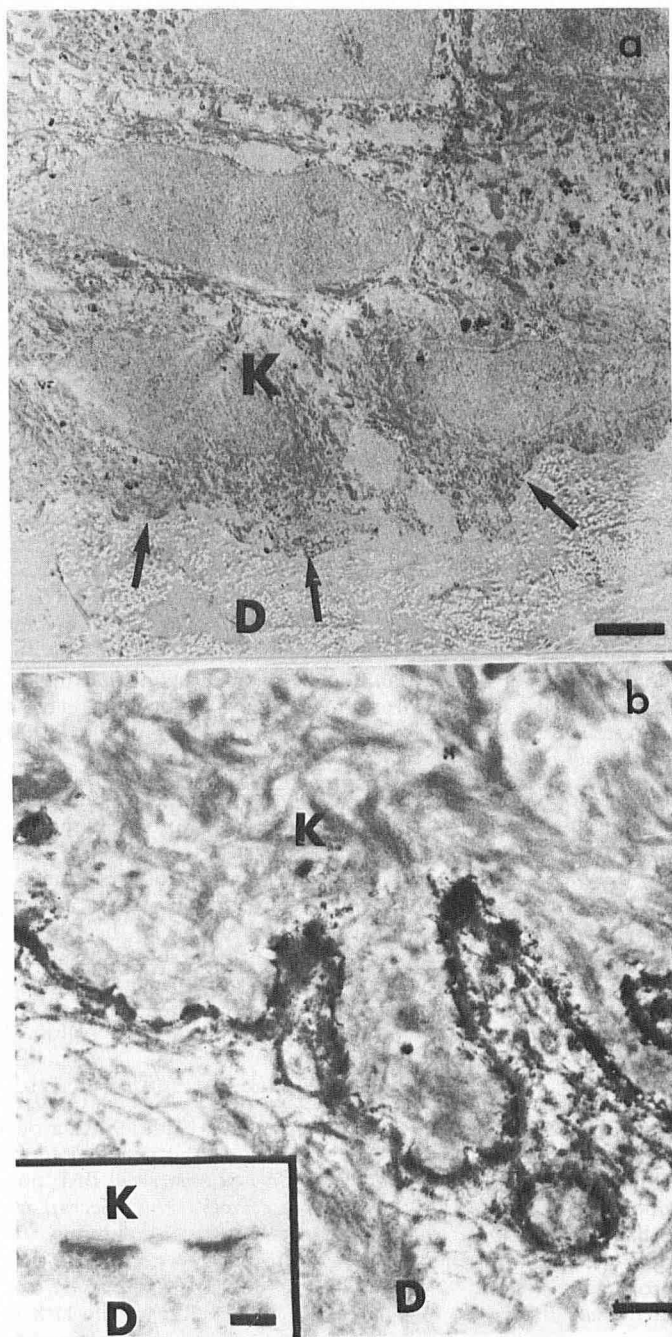
**Bidimensional Gel Electrophoresis Analysis:** In this preliminary set of experiments, 2D gel electrophoresis of cultured keratinocyte proteins was carried out in order to better characterize the 37–38 kD doublet. In Fig 7, Coomassie blue staining of the protein is shown. Corresponding blotting sheets with nonimmune and 6/2 sera is evidenced in Fig 8. It is clearly seen that in the range of 37–38 kD, there is not a unique spot, but several spots related to charge heterogeneity of the antigen. A major form at 37 kD with an apparent pI<sub>i</sub> of approximately 7.3 is detected which is followed by other species with higher molecular weight and more acidic charges.

**Antigen Recognized by 6/2 Antiserum Is Not Related to Laminin, Fibronectin, and Collagen IV** Although the molecular weight of antigen recognized by 6/2 antiserum is quite different from other known components of epidermal BM, possible cross-reactivity of the serum with laminin, fibronectin, and collagen IV was checked by immunoblotting. Antilaminin, antifibronectin, and anticollagen IV sera were reacted against their proper antigen which was previously run on SDS-PAGE, and then transferred to a nitrocellulose sheet. In Fig 9, the lack of cross-reactivity of 6/2 antiserum with any of these proteins on nitrocellulose sheet is clearly evidenced, whereas these antigens react with their respective antibodies giving characteristic bands.

#### DISCUSSION

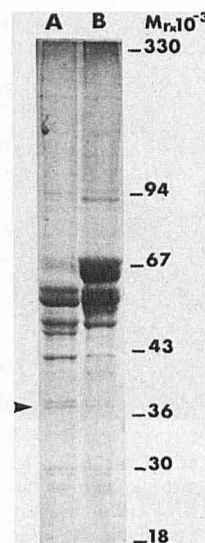
This study demonstrates that a shared antigen was identified between the amniotic epithelial basement membrane (AEBM) and at the BM of the skin by using a rabbit anti-AEBM serum called 6/2 serum. In view of the common ectodermal ancestry of amniotic epithelium and the major epithelial components of skin, it is not surprising to find that both AEBM and epidermal BM contain laminin, collagen IV, and probably fibronectin [2,19]. However, as determined by indirect IF and immunoblot analysis, it is clear that the antigen detected by 6/2 serum is different from laminin, collagen IV, and fibronectin. Unlike laminin and collagen IV which are ubiquitous components of BM, 6/2 antigen is not present at the BM zones of blood vessels or nerves.

The results on suction blisters by indirect IF and those on normal human skin by immunoelectron microscopy indicate that



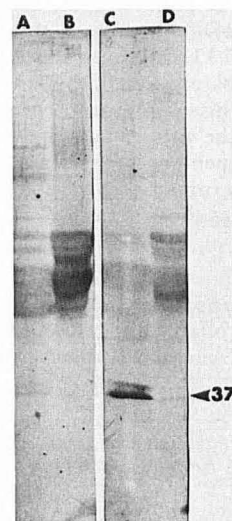
**Figure 4.** The reactivity of 6/2 serum on normal human skin by immunoelectron microscopy. Preparations were not counterstained. *a*, Control. Nonimmune serum. *K* = keratinocytes, *D* = dermis, arrows = dermal-epidermal junction ( $\text{bar} = 2 \mu\text{m}$ ). *b*, The reactivity is observed at the dermal-epidermal junction. *K* = keratinocytes, *D* = dermis ( $\text{bar} = 500 \text{ nm}$ ). Inset, Higher magnification. The staining is located at the level of the lamina lucida. *K* = keratinocytes, *D* = dermis ( $\text{bar} = 100 \text{ nm}$ ).

the antigen is localized at the lamina lucida of human BM. However, the presence of this antigen at lamina densa cannot be firmly ruled out based on the findings that a strong reactivity was observed at the blister floor by IF (Fig 2c) and the weak labeling of blister roof by immunoblotting (Fig 6). However that may be, immunoelectron microscopy shows that the antigen is found in hemidesmosomes (extracellular part). The data of normal human keratinocyte culture suggest that the antigen recognized by 6/2 serum is probably produced *in vivo* by keratinocytes. This hy-

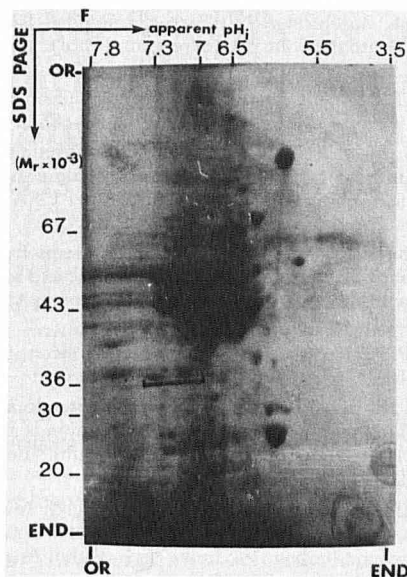


**Figure 5.** SDS-PAGE of cultured human keratinocytes (*A*) and blister roof epidermis (*B*). Proteins were separated and stained with Coomassie blue as described in *Materials and Methods* on a 10% acrylamide gel. The molecular weights ( $M_r \times 10^{-3}$ ) were calculated from the migration of standard proteins. Arrowhead indicates the 37 kD band of the 37–38 kD doublet. Note the absence of 65–67 kD cytokeratins in cultured cells.

pothesis is strengthened by the presence of strong cytoplasmic reactivities of some keratinocytes which were usually in contact with the collagen coat (Fig 3). This is not too unreasonable if one considers that all the components of the lamina lucida are of epidermal origin, and are produced by keratinocytes [2]. Furthermore, it is supported by the results that the antigen was detected beneath the migrating keratinocytes very early during wound healing of the skin. It is interesting to note that the kinetics



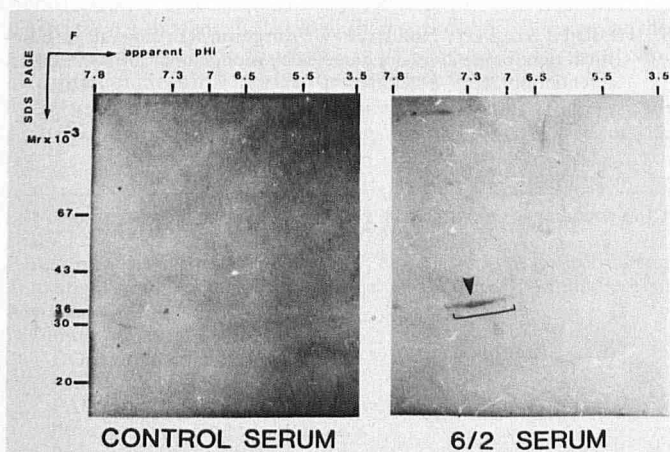
**Figure 6.** Immunoblot analysis of proteins from cultured keratinocytes (*A* and *C*) and from blister roof epidermis (*B* and *D*) with 6/2 serum. This figure shows the pattern of proteins electrophoretically transferred onto nitrocellulose sheets after they were reacted with sera and revealed with the peroxidase method. *A* and *B*, Transferred proteins reacted with non-immune rabbit serum diluted 1:200 (control). *C* and *D*, Transferred proteins reacted with 6/2 rabbit serum diluted 1:20 (because this serum contains 10-fold less protein than normal serum—see *Materials and Methods*). Arrowhead indicated molecular weight ( $\times 10^{-3}$ ) of the major protein of the 37–38 kD doublet present in cultured keratinocytes (*C*) but absent in blister roof epidermis (*D*).



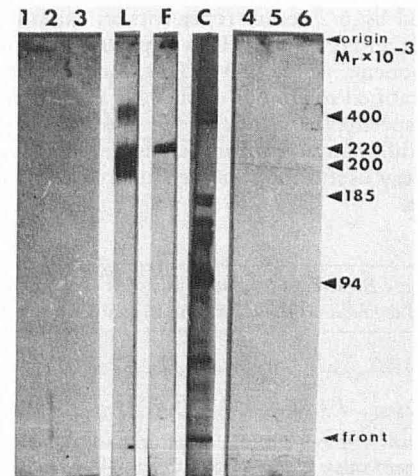
**Figure 7.** Two-dimensional gel electrophoresis of cultured human keratinocytes. Coomassie blue staining of cell proteins separated in the first dimension according to apparent isoelectric focusing (F) and in the second dimension according to SDS-PAGE on a 10% acrylamide gel. Bracket indicates the position of the 37–38 kD doublet identified on monodimensional gels. The major form at 37 kD is essentially seen at 7.3 p*H*<sub>i</sub>. OR = origin; END = end of the protein migration on SDS-PAGE. At the center of the gel, note the abundance of cytokeratins.

of antigen that binds 6/2 serum closely parallels that of BP antigen [20,21], but it is quite different from those of collagen IV and laminin that do not reappear until the wound is covered and the epidermis becomes stationary. Although the role of the antigen evidenced in this study is unknown, these observations suggest that it plays a critical role in the establishment of integrity between epidermal cells and the wound matrix and perhaps in the process of immobilization of migrating keratinocytes.

Biochemical characterization of the antigen recognized by 6/2 serum in keratinocyte culture shows that it is a protein that exists as a major form at 37 kD and an apparent p*H*<sub>i</sub> = 7.3, but that isoforms or species with more acidic charges may also be present in the range of 37–38 kD. This could explain the



**Figure 8.** Two-dimensional immunoblot analysis of proteins from cultured keratinocytes with 6/2 serum. Bracket indicates the multi-spots-streak of 37–38 kD identified specifically by 6/2 serum. The protein with an apparent p*H*<sub>i</sub> = 7.3 at 37 kD is the major form of the antigen (arrowhead). F = first dimension according to apparent isoelectric focusing. Control serum: nonimmune rabbit serum.



**Figure 9.** Immunoblot cross-reactivity of 6/2 serum with laminin, fibronectin, and collagen IV. 1–3, Nonimmune rabbit serum reactivity with laminin (L), fibronectin (2), and collagen IV (3) antigens. L, Anti-laminin serum reactivity with laminin. F, Antifibronectin serum reactivity with fibronectin. C, Anticollagen IV serum reactivity with collagen IV. 4–6, 6/2 serum reactivities with laminin (4), fibronectin (5), and collagen IV (6) antigens. Electrophoresis of antigens was performed on 5% acrylamide gels.

doublet at 37–38 kD observed on immunoblot from monodimensional SDS-PAGE of cell proteins. This doublet cannot be related to degradation products of a main protein, because protease inhibitor (PMSF) was added to the homogenate buffer. Furthermore, during the short time of recovering the cells, all operations were performed at 4°C to reduce hydrolysis. The finding that species with charge differences seem to be more or less abundant depending on keratinocyte culture series is yet unexplained but, at present, under active investigation.

It should be stressed that this antigen shared several common features with BP antigen. Both are present at the lamina lucida of the BM zone of mammalian stratified squamous epithelia including epidermis (apparently related to hemidesmosomes [22,23]), mucosa of the oral cavity, upper esophagus, vagina, and cornea. In addition, BP sera gave, by IF technique, a linear staining at the BM zone of reflected and placental amnion, as well as the amnion covering the umbilical cord [6]. There are, however, major differences between these antigens. By immunoprecipitation studies, the BP antigen derived from epidermal cell culture has been identified as a 220 kD protein [24], whereas by immunoblots, 6/2 serum bound an antigen of 37 kD. This does not rule out the possibility that this antigen may be a subunit of BP antigen. However, blocking the expression of the 37 kD antigen could not be accomplished by the preincubation with BP serum or vice versa. In addition, it has recently been shown that 6/2 serum detected an abnormality of the dermal-epidermal junction in a disorder called junctional epidermolysis bullosa, in which the expression of BP antigen was normal [25]. It is tempting to suggest that the 37 kD antigen could be involved in the pathogenesis of herpes gestationis, a disease of the dermal-epidermal junction associated with pregnancy [26]. The antigen involved in herpes gestationis is so far unknown, but from the blocking experiments, it may be concluded that it is distinct from the 37 kD antigen.

Recently, a series of GB monoclonal antibodies to human amnion were developed [27]. Among them, GB3 reacted with AEBM and BM in the dermal-epidermal junction, as well as certain ectoderm-derived epithelial BM. Its reactivity is similar to that of monoclonal antibody 161 which was prepared against human amniochorion [28]. However, it is difficult to compare the 2 antibodies simply by tissue distribution; precise identity must await further biochemical characterization. In conclusion, the an-



tigen identified by 6/2 serum represents an example of the BM diversity. This antigen of 37 kD is apparently distinct from the known components of the BM. This antigen is expressed by simple and stratified squamous epithelia of ectodermal origin. Its wide species specificities allow its use not only on human, but also on many different types of animal tissues. Finally, this marker should be a very useful tool for the study of BM and the extracellular matrix.

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